UVB-Induced Melanocyte Proliferation in Neonatal Mice Driven by CCR2-Independent Recruitment of Ly6c^{low}MHCII^{hi} Macrophages

Herlina Y. Handoko[1,4], Mathieu P. Rodero[2,4], Glen M. Boyle[1], Blake Ferguson[1], Christian Engwerda[1], Geoff Hill[1], H. Konrad Muller[3], Kiarash Khosrotehrani[2,5] and Graeme J. Walker[1,5]

Intermittent sunburns, particularly in childhood, are the strongest environmental risk factor for malignant melanoma (MM). In mice, a single neonatal UVR exposure induces MM, whereas chronic doses to adult mice do not. Neonatal UVR alters melanocyte migration dynamics by inducing their movement upward out of hair follicles into the epidermis. UVR is known to induce inflammation and recruitment of macrophages into the skin. In this study, we have used a liposomal clodronate strategy to deplete macrophages at the time of neonatal UVR, and have shown functionally that this reduces the melanocyte proliferative response. This effect was not reproduced by depletion of CD11c-expressing populations of dendritic cells. On the basis of epidermal expression array data at various time points after UVR, we selected mouse strains defective in various aspects of macrophage recruitment, activation, and effector functions, and measured their melanocyte UVR response. We identified Ly6c^{low}MHCII^{hi} macrophages as the major population promoting the melanocyte response across multiple strains. The activity of this subpopulation was CCR2 (C-C chemokine receptor type 2) independent and partly IL-17 dependent. By helping induce this effect, the infiltration of specific macrophage subpopulations after sunburn may be a factor in increasing the risk of subsequent neoplastic transformation of melanocytes.

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INTRODUCTION

Epidemiological studies clearly show that exposure of the skin to UVR can increase one's risk of developing malignant melanoma (MM) (Gandini *et al.*, 2005). Indeed, MM risk is strongly associated with geographical location, with incidence rates for white-skinned populations in regions of high UVR flux at the highest risk (Whiteman and Green, 2005). Protection from sun exposure with sunscreen can significantly reduce risk (Green *et al.*, 2011). However, the link between UVR and MM is complex. Meta-analysis of all epidemiological studies that assessed sun exposure and MM risk showed that MM development is overwhelmingly influenced by intermittent exposures (Gandini *et al.*, 2005), particularly childhood sunburns (Whiteman *et al.*, 2001). This is in contrast to epidermal keratinocyte-based skin cancers such as squamous cell carcinoma, which is driven by cumulative UVR exposure in a dose-dependent manner (Rigel, 2008). Hence, the mechanisms that govern UVR-induced melanoma genesis may be more complex than simply the induction and accumulation of mutations through genotoxicity.

To study the mechanisms of UVR-induced melanoma genesis, murine models prone to the development of the neoplasm are commonly used. It is well known that a single relatively high-dose UVB exposure to these neonatal mice is effective in inducing MM in mice, whereas even chronic doses to adult mice are not (Noonan *et al.*, 2001; Hacker *et al.*, 2005), mimicking the epidemiological observations in humans. Mice are nocturnal species, with most of their skin covered by fur. In adult mice, melanocytes are almost confined to the hair follicle (except in the ears and tail where they are occasionally found in the epidermis), and melanin production is strictly coupled to anagen phase of hair growth. However, in newborn murine skin, melanocytes are present in the epidermis for the first week of life (Hirobe, 1984).

The regulation of melanocyte function within the skin and hair follicle is complex, with potentially both local and systemic factors at play, and melanocytes themselves may even have a role in skin homeostasis (Slominski *et al.*, 2004,

[1]*Queensland Institute of Medical Research, Brisbane, Queensland, Australia;* [2]*Experimental Dermatology Group, The University of Queensland, UQ Centre for Clinical Research, Herston, Brisbane, Queensland, Australia and* [3]*School of Medicine, University of Tasmania, Hobart, Tasmania, Australia*

[4]*The first two authors contributed equally to this work.*

[5]*The last two senior authors contributed equally to this work.*

Correspondence: Kiarash Khosrotehrani, University of Queensland Centre for Clinical Research, Royal Brisbane Hospital, Building 71/918, Herston, Brisbane, Queensland 4029, Australia.
E-mail: k.khosrotehrani@uq.edu.au; Graeme J. Walker, Queensland Institute of Medical Research, 300 Herston Road, Herston, Brisbane, Queensland 4006, Australia. E-mail: Graeme.Walker@qimr.edu.au

Abbreviations: CCR2, C-C chemokine receptor type 2; MHCII, major histocompatibility complex class II; MM, malignant melanoma; MyD88, myeloid differentiation primary response gene (88); PBS, phosphate-buffered saline; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; TRIF, TIR-domain-containing adapter-inducing interferon-β; WT, wild type

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2005; Plonka *et al.*, 2009). Interestingly, in addition to the activation of pigmentation pathways in melanocytes, UVR exposure produces an increase in epidermal basal layer melanocyte numbers in both humans and mice (Quevedo *et al.*, 1965; Stierner *et al.*, 1989; Yamaguchi *et al.*, 2008). Increased numbers of melanocytes are observed around the hair follicle outer root sheath in human skin that has been exposed to chronic UVR (Staricco and Miller-Milinska, 1962). As the increase in melanocyte number probably helps to increase the total amount of melanin produced, this proliferative response of melanocytes may be linked to, although not necessary for, the tanning process. We previously reported that neonatal mice have an exquisite sensitivity to UVR-induced melanocyte proliferation and migration, leading to a large increase in epidermal melanocyte numbers compared with untreated control skin (Walker *et al.*, 2009). Thus, notwithstanding the differences between murine and human skin in terms of epidermal thickness and the density of hair follicles, they provide a tractable system to investigate the mechanisms of the melanocyte UVR response, and also, using appropriate mouse models, to determine whether this response is important for MM induction.

Keratinocytes have been suspected to have an important role in the melanocyte response to UVR, as they can produce many cytokines and growth factors that can evoke melanocyte proliferation in cell culture. These include KITL, EDN1, bFGF, HGF, LIF, $PDGE_2$, GM-CSF, and Wnt ligands (Hirobe, 2005; Rabbani *et al.*, 2011), many of which are capable of influencing both the melanocytic and immune systems. In addition to its effect on keratinocytes, UVR-induced inflammation may influence the melanocyte response. It has long been known that macrophages enter the skin after chronic or acute sun exposure. Meunier *et al.* (1995) reported that macrophages increase in number over 2-fold in the human dermis after a single sunburn. They appeared to be derived from both transcapillary migration (i.e., recruitment) and expansion of preexisting monocyte precursors in the dermis. Aoki and Moro (2005) suggested that UVR-induced inflammation could be driving melanocyte proliferation. Their studies on chronic UVR-induced pigmented spots in hairless mice led them to hypothesize that chemokines produced in the skin attract inflammatory cells to the vicinity of melanocytes, providing an interactive network and environment for melanocyte activation. The potential role for the inflammatory response in driving melanocyte proliferation response to UVR has been further highlighted by Zaidi *et al.* (2011). In this case, macrophages were suspected to be the origin of excess IFN-γ production affecting melanocyte proliferation.

Here we hypothesized that macrophages have a central role in inducing melanocyte proliferation and/or migration to the areas of skin exposed to UVR. In newborn mice, in which UVB significantly exacerbates MM development, we have used a macrophage depletion strategy to verify this, and have identified $Ly6c^{low}MHCII^{hi}$ macrophages as the leading population in promoting the melanocyte response. We determined that the macrophage promotion of the melanocyte response is not driven by classical inflammatory, but by ''alternatively activated'' noninflammatory macrophages whose infiltration is mostly CCR2 (C-C chemokine receptor type 2) independent. By inducing melanocyte proliferation in response to UVR, macrophages may have an important role in the early processes involved in the initiation of MM.

RESULTS

Increase in epidermal melanocytes is concomitant with influx of F4/80-positive monocytes

Newborn mice were irradiated with 5.9 kJ m^{-2} UVB at 3 days of age (P3), and the skin was harvested 4 days (D4) after UVR (P7), representing the peak of melanocyte response according to our previous findings (Walker *et al.*, 2009) (Figure 1a). A control group consisted of nonirradiated or shielded littermates. Skin sections harvested at D4 (Figure 1b and c) show a dense infiltrate of F4/80-positive monocytic cells in the exposed skin and not in the shielded skin. These F4/80-positive cells include Langerhans cells that, as expected, have moved out of the epidermis by D4 in the exposed skin. To detect melanocytes, skin sections were stained with an anti-c-KIT antibody (Figure 1e and f). We used c-KIT because it marks epidermal and follicular melanocytes at an earlier state of differentiation than most pigmentation markers (see, e.g., Grichnik *et al.*, 1996; Sharov *et al.*, 2003); hence, by using c-KIT we are less likely to miss any relatively immature cells in the epidermis. Notably, although c-KIT stains mast cells and a subpopulation of keratinocytes in the lower hair bulb (Peters *et al.*, 2003), it does not stain epidermal keratinocytes. The c-KIT-staining epidermal melanocytes that we counted can be clearly differentiated from these other cell types, and are consistent in shape and location as the tyrosinase-related protein 1 and 2-positive cells that we previously reported that increase in the epidermal basal layer after neonatal UVR (Walker *et al.*, 2009).

The numbers of melanocytes per mm^2, with and without UVR, can be seen in Figure 1g. In the shielded skin, almost no melanocytes could be seen at the epidermal basal layer, although they could be found in deeper areas of the hair follicles. At D4, however, highly dendritic melanocytes can be seen lining the permanent (upper) portion of the hair follicle and the epidermal basal layer at a density of 55.25 (±1.35) cells per mm^2.

The phenotype of skin-infiltrating macrophages changes over time

To investigate the makeup of the inflammatory infiltrate, we used flow cytometry to assess the quantity and phenotype of monocytes in the skin at various time points after UVR. Macrophage numbers increased until D4 in absolute numbers. By D7, we could not detect any macrophages in the skin using flow cytometry (Supplementary Figure S1a and b online). Hence, the macrophage response to UVR shows similar kinetics to the melanocyte response, both peaking at D4 after UVR and then diminishing. Costaining of UVR-exposed skin sections with F4/80 and Ki67 did not suggest proliferation of macrophages locally, but that the increase is likely to be via recruitment. Ly6c expression by macrophages has been used to evaluate the inflammatory status of a mouse tissue

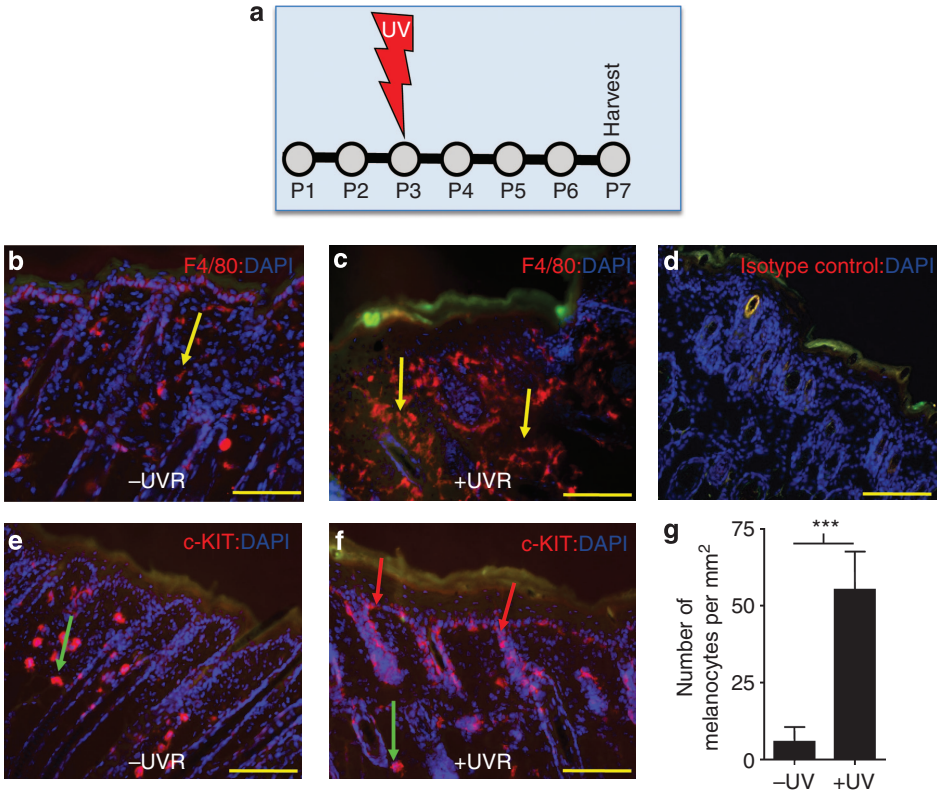

Figure 1. Concurrent increase in epidermal melanocytes and dermal macrophages after neonatal UVB irradiation. (**a**) Schematic representation of our protocol of the neonatal irradiation. After harvest, skin was stained with F4/80 or c-KIT antibodies (red) and 4′,6-diamidino-2-phenylindole (DAPI; blue). Control (−UVR) = shielded skin, +UVR = exposed skin of the same animal. (**b**, **c**) Yellow arrows indicate monocytic cells in the dermis. F4/80-positive Langerhans cells can be seen in the epidermis of nonexposed skin. (**d**) Isotype control using IgG2 secondary antibody (same for both c-KIT and F4/80). (**e**, **f**) c-KIT-positive melanocytes (red arrows) can be seen at the epidermal basal layer in exposed but not shielded skin. Mast cells are seen as round red cells (green arrows) in the dermal part of the skin. (**g**) The number of melanocytes in the skin per microscope field, with and without UVB exposure, 4 days after UVR (D4). $N = 20–50$ fields from at least 12 pups obtained from independent experiments. Result shown as mean ± SD. ***$P < 0.001$. Scale bars = 100 μm. Mouse strain background was C57BL/6J.

(Nahrendorf *et al.*, 2007; Cochain *et al.*, 2010). From D2, Ly6c^{low} ''non-inflammatory'' macrophages represented the dominant population, whereas both Ly6c^{low} and Ly6c^{hi} monocytes/macrophages were represented at earlier time points (Supplementary Results and Supplementary Figure S1c and d online).

Major histocompatibility complex class II (MHCII) is a commonly expressed marker with high heterogeneity on monocytes and macrophages, and it is highly expressed in macrophages infiltrating human skin after UVR (Meunier *et al.*, 1995; Ingersoll *et al.*, 2010). Indeed, in UVR-irradiated mouse skin, MHCII levels discriminated Ly6c^{hi} and Ly6c^{low} macrophages into subpopulations (Figure 2a, second and third panels, and Figure 2b and c). We observed a specific increase of the Ly6c^{low}MHCII^{hi} population between D2 and D4 (from 16.72 to 40.79%, $P = 0.0001$; Figure 2d). This increase was concomitant with the melanocyte response peaking at D4 (Walker *et al.*, 2009), suggesting a possible link between the two populations. Using CD11c-DTR/GFP mice, we established the level of expression of CD11c by each of the four skin-infiltrating macrophage populations (Supplementary Figure S2 online). In all, 50% of the Ly6c^{low}MHCII^{hi} cells expressed CD11c at least at a low level.

Macrophage depletion reduces UVR-induced melanocyte proliferation/migration

To test whether infiltrating macrophages influence melanocyte proliferation or migration *in vivo*, we measured epidermal melanocyte numbers after macrophage depletion using clodronate-filled liposomes (with phosphate-buffered saline (PBS)-filled liposomes as controls). To deplete both the local and systemic recruited subpopulations, liposomes were injected both intraperitoneally and subcutaneously at 1 day before UVR (D − 1), on the day of UVR (D0), and at day 2 (D2) after UVR (Figure 3a). As expected, the clodronate-treated group showed a significant reduction in macrophage numbers in the skin, although neutrophils were not affected, excluding any off-target effect (Figure 3b). On the basis of Ly6c and MHCII expression, all macrophage populations were affected by this depletion, although the Ly6c^{low}MHCII^{low} population was less depleted in proportion than the others (Supplementary Figure S3 online). Most interestingly, in UVB-irradiated neonates subjected to macrophage depletion, we observed a significant reduction in the number of interfollicular epidermal melanocytes per mm^2 compared with that for controls injected with PBS (i.e., 32.5 (±1.45) vs. 51.5 (±2.45; $P < 0.0001$; Figure 3c and d). Clodronate treatment of

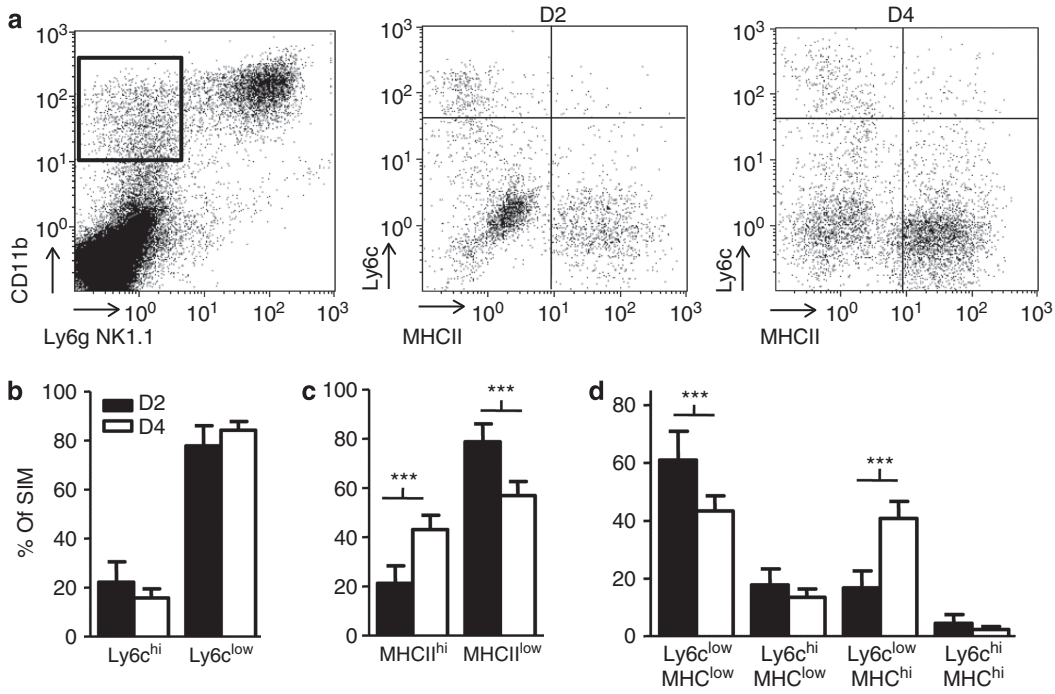

Figure 2. Major histocompatibility complex class II (MHCII) and Ly6c expression levels define skin macrophage populations with different infiltration patterns. (**a**) The first panel shows gating of CD11b^{hi}/Ly6g^{low} macrophages. The second and third panels show the relative proportions of the macrophage populations based on Ly6c and MHCII staining at day 2 (D2) and day 4 (D4) after UVR, respectively. (**b**) Representation of the proportion of macrophages based on Ly6c. SIM, skin-infiltrating macrophage. (**c**) MHCII or (**d**) both Ly6c and MHCII levels of expression. $N = 8$–9 pups for each time point and is representative of two independent experiments. Results shown as mean ± SD. ***$P < 0.001$.

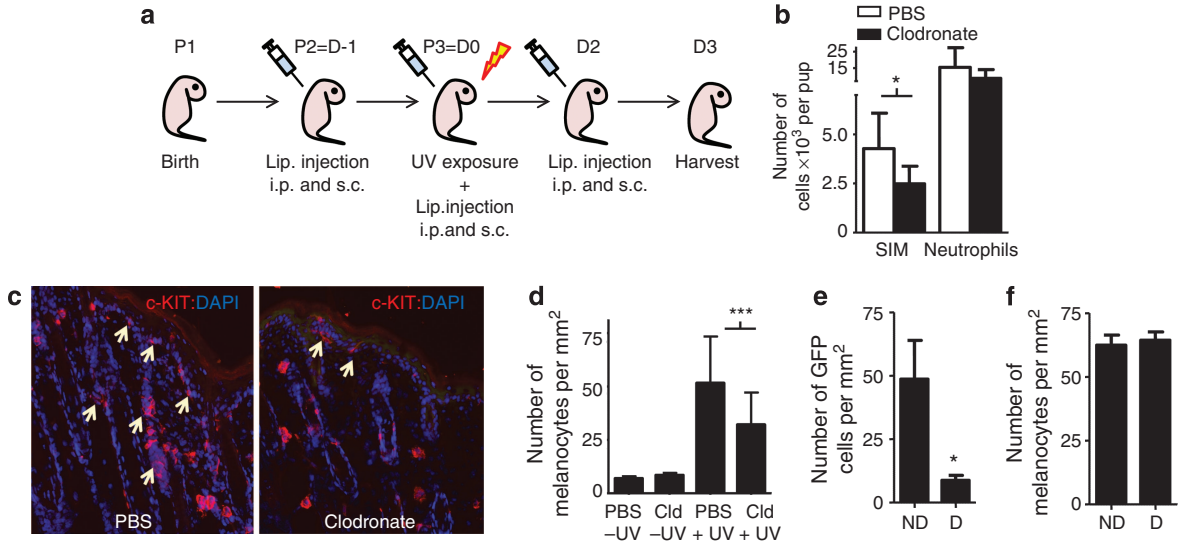

Figure 3. Macrophage depletion but not CD11c$^+$ cell depletion decreases melanocyte response to UVR. (**a**) Schematic protocol of irradiation and injection of clodronate to neonates. (**b**) Representation of the effect of clodronate injection on macrophages and neutrophils. (**c**) More c-KIT-positive melanocytes (white arrows) in control UVB-irradiated skin, compared with irradiated skin treated with clodronate. (**d**) Melanocyte counts per field in UVB-irradiated skin (no UVR for control), with clodronate or phosphate-buffered saline (PBS) treatment. $N = 5$–8 pups for each group from two independent experiments. CD11c-DTR/GFP mice were injected with diphtheria toxin or PBS at 1 day before UVR (D − 1), on the day of UVR (D0), and at day 2 (D2) after UV exposure. Skin was collected at day 3 (D3). (**e**) Number of green fluorescent protein (GFP) cells to show the depletion dendritic cells. (**f**) Melanocyte response was evaluated in depleted ($n = 3$) and control ($n = 5$) mice. Results represent pooled data from two independent experiments. Cld, clodronate; D, depleted, DAPI, 4′,6-diamidino-2-phenylindole; i.p., intraperitoneal; ND, nondepleted; Lip., liposome; s.c., subcutaneously; SIM, skin-infiltrating macrophage. Result shown as mean ± SD. *$P < 0.05$; ***$P < 0.001$.

neonates not exposed to UVR did not reduce melanocyte number, showing that clodronate did not directly affect melanocytes. As clodronate liposomes target all highly phagocytic populations, they also depleted populations of dendritic cells. We therefore used CD11c-DTR/GFP mice (Jung *et al.*, 2002) to assess the role of dendritic cells in the

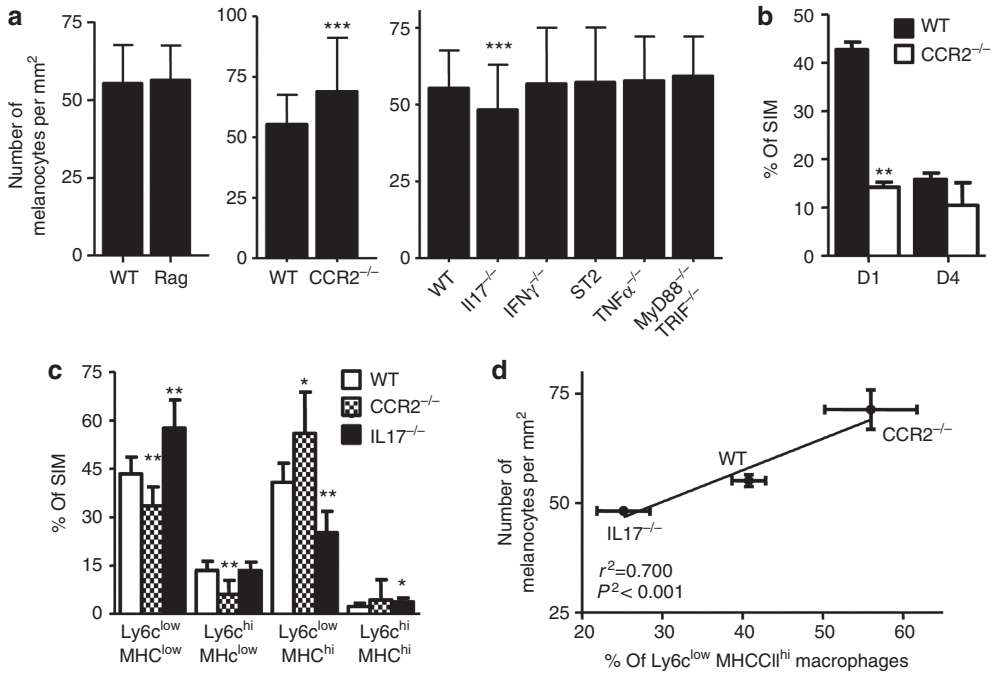

Figure 4. Ly6c^{low}MHCII^{hi}macrophages promote melanocyte response to UVR. (**a**) The number of melanocytes per field in UVB-exposed skin from wild-type (WT) and mice deficient for RAG1, CCR2, IL-17, TNF-α, IFN-γ, ST2, or MyD88/TRIF at day 4 (D4) after exposure. CCR2, C-C chemokine receptor type 2; MyD88, myeloid differentiation primary response gene (88); TNF-α, tumor necrosis factor-α; TRIF, TIR-domain-containing adapter-inducing interferon-β. (**b**) Proportion of Ly6c^{hi} among macrophages in the skin at day 1 (D1) and D4 after exposure in WT and *CCR2*$^{-/-}$ mice. (**c**) Proportion of skin-infiltrating macrophage (SIM) populations based on Ly6c and major histocompatibility complex class II (MHCII) levels of expression at D4 after exposure in WT, *CCR2*$^{-/-}$, and *IL17*$^{-/-}$ mice. (**d**) Correlation between the proportion of Ly6c^{low} MHCII^{hi} macrophages and the melanocyte response at D4 after exposure in WT, *CCR2*$^{-/-}$, and *IL17*$^{-/-}$ mice. **a**, $N = 22$–96 fields from a minimum of 3 pups; **b**, $N = 4$–8 pups and is representative of two independent experiments; and **c**, $N = 5$–8 pups from two independent experiments with a similar trend. Results shown as mean ± SD. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

context of UVR and melanocyte response. Despite efficient depletion in neonates (significant reduction in the number of GFP+ cells per field), the epidermal melanocyte density after UVR was similar to neonates that were not depleted (Figure 3e and f).

Characterization of the UVB-induced inflammation by expression profiling

Having confirmed the role of UVR-induced macrophages in the skin in promoting melanocyte proliferation, we next addressed the question of potential mechanisms. To ask what may be released in the epidermis after UVR to attract macrophages into the skin, we performed gene expression array analysis on UVR-treated epidermis. Melanocyte numbers increase at D2 after UVR (Walker *et al.*, 2009); therefore, the necessary stimulus is probably present much earlier. Thus, we selected 6, 10, and 24 hours, and D3 after UVR, using three pups per time point. As gene expression in the neonatal epidermis is dynamic over time even without UVR, we used shielded skin as control (i.e., each mouse is its own control). Thus, our results are not skewed by normal developmental gene expression changes that occur in the pups over the 72-hour period (detailed in Supplementary Results and Supplementary Table S1 online).

Examination of earlier time points (6, 10, and 24 hours after UVR) revealed the potential role of immune-related genes. Defensins (*DEFβ4* and *DEFβ14*) showed a significant upregulation after UVR. *DEFβ4* has been shown to induce macrophage infiltration, possibly by binding the macrophage CCR2 or Toll-like receptors (TLRs) (Röhrl *et al.*, 2010). In human epidermis, β-defensin is upregulated over 50-fold after UVR exposure (Enk *et al.*, 2006), and in the context of melanocyte activation it may be another ligand (in addition to agouti signaling peptide (ASIP)) for the melanocortin 1 receptor (MC1R), a receptor whose deregulation is important in melanoma (Candille *et al.*, 2007; Beaumont *et al.*, 2012; Swope *et al.*, 2012). Interestingly, other cytokines were significantly transcriptionally upregulated in the neonatal skin by UVR exposure, including IL-33 and tumor necrosis factor-α (TNF-α). The former has been shown to induce inflammatory macrophage infiltration (Kurowska-Stolarska *et al.*, 2009). In summary, despite the limitations of gene expression array studies on mixed cell populations in the epidermis, our results point to major changes in immune regulation in exposed skin.

CCR2-negative Ly6c^{low}MHCII^{hi} macrophages promote the melanocyte response in part through IL-17

We then set out to further dissect the molecular mechanism of the promotion of the neonatal melanocyte response by macrophages. In light of our expression array findings, we studied melanocyte behavior in mice deficient for key cells

and molecules involved in the inflammation process. First, we measured the melanocyte response in *RAG1*$^{-/-}$ mice and found it to be the same as in wild-type (WT) mice (C57Bl/6J), essentially ruling out a significant role of T or B lymphocytes in this response (Figure 4a).

We next considered CCR2, a major chemokine receptor involved in macrophage recruitment. Surprisingly, the number of epidermal melanocytes was higher in the *CCR2*$^{-/-}$ mice than in WT pups (68.8 vs. 55.25, $P<0.0001$; Figure 4a). Furthermore, the total number of skin-infiltrating macrophages was not decreased in *CCR2*$^{-/-}$ mice compared with WT at any time point after UVR, as measured by either flow cytometry or F4/80 staining of skin sections. However, flow cytometry analyses at D1 after UVR revealed a marked defect in the recruitment of the initial Ly6c^{hi} inflammatory population, known to be CCR2-positive cells (Geissmann *et al.*, 2003), in the *CCR2*$^{-/-}$ mice compared with WT (Figure 4b). By D4, the proportion of Ly6c^{hi} and Ly6c^{low} macrophages was the same between WT and *CCR2*$^{-/-}$ mice, and the macrophage infiltration into the skin in response to UVR mainly comprised the CCR2-independent cell population.

Defensins have been described to activate myeloid cells through TLRs (Biragyn *et al.*, 2008; Semple *et al.*, 2011). We therefore postulated that the regulation of the melanocyte response by skin-infiltrating macrophages might be MyD88/TRIF (myeloid differentiation primary response gene (88)/TIR-domain-containing adapter-inducing interferon-β) dependent as the main downstream signaling molecules associated with TLRs. Similarly, the increased expression of *TNFα* and *IL33* prompted us to consider their role in the activation of macrophages and therefore in the melanocyte response. Consequently, we compared the melanocyte response in WT, ST2 (*IL33R*$^{-/-}$), *TNFα*$^{-/-}$, and *MyD88/TRIF*$^{-/-}$ mice (Figure 4a). The absence of any difference in epidermal melanocyte counts clearly showed that none of these pathways/molecules alone was important for the melanocyte response to UVR.

Finally, we considered inflammatory cytokines usually produced by effectors such as IL-17 and IFN-γ. The significant increase in β-defensin, as well as *S100A8* or *S100A9* transcription in the epidermis, after UVR could reflect a response to IL-17, as previously described in the lung and skin epithelia (Kao *et al.*, 2004; Kryczek *et al.*, 2008). In accordance, *IL17*$^{-/-}$ mice showed a 15% decrease in the number of interfollicular epidermal melanocytes when compared with WT mice, suggesting a role for IL-17 possibly in the melanocyte response to UVR. We did not observe any significant effect in IFNγ$^{-/-}$ mice.

We next analyzed, by flow cytometry, the macrophage subpopulations infiltrating the skin in the IL17$^{-/-}$, CCR2$^{-/-}$, and WT mice at D4 after UVR (Figure 4c). Surprisingly, in *CCR2*$^{-/-}$ mice, despite the marked reduction in CCR2-dependent ''inflammatory'' infiltrate at 24 hours, the proportion of Ly6c^{low}MHCII^{hi} macrophages was higher at 4 days after UVR compared with WT (1.37-fold; 56 ± 12.8 vs. 40.8 ± 5.9%). In contrast, it was significantly decreased in *IL17*$^{-/-}$ mice, by 0.61-fold (25.2 ± 6.6%; $P<0.05$ for all comparisons with WT using Mann–Whitney test). In addition, linear regression analysis revealed a strong positive correlation between the melanocyte response and the proportion of the Ly6c^{low}MHCII^{hi} macrophages in WT, *IL17*$^{-/-}$, and *CCR2*$^{-/-}$ mice (Figure 4d; $r^2=0.69$, $P<0.001$), further suggesting that Ly6c^{low}MHCII^{hi} cells contribute to the melanocyte response.

DISCUSSION

The neonatal period is critical in terms of inducing MM in mice using UVR (Noonan *et al.*, 2001). Exposure during this period induces a melanocyte proliferative and migratory response, as reflected by their presence in the interfollicular epidermis and the upper portion of the hair follicle. Suspecting an important role for the inflammatory component of the UVR response in driving melanocyte proliferation and migration, we have described the kinetics of macrophage infiltration into the skin using multicolor flow cytometry. We then used a macrophage depletion strategy to demonstrate functionally the existence of a macrophage–melanocyte link. Using a number of mouse models with various inflammatory defects, we established that this melanocyte response is dependent upon infiltrating CCR2-negative macrophages and, in part, dependent on IL-17.

Macrophages are known to be a heterogeneous population that increases in size partly from the recruitment of circulating monocytes to sites of inflammation. During macrophage infiltration, a transition from mainly Ly6c^{hi} to mainly Ly6c^{low} cells has been proposed to reflect the resolution of the inflammation (Geissmann *et al.*, 2003; Nahrendorf *et al.*, 2007; Cochain *et al.*, 2010). In our model, the recruited Ly6c^{hi} macrophages reached a peak at D1 after UVR and returned to their steady-state proportion at D2. This would support the notion of an early classical inflammatory response after neonatal UVR via recruitment of Ly6c^{hi} CCR2–positive monocytes that mostly ceases after D1. This early transition from Ly6c^{hi} to Ly6c^{low} within the first 24 hours seems to be specific to UVR when compared with other inflammatory situations. However, the number of macrophages continued to increase between D1 and D4 without significant proliferation, suggesting additional recruitment of mostly Ly6c^{low} cells, reported to be CCR2 negative (Geissmann *et al.*, 2003). *In vivo*, macrophages are reported to be heterogeneous, and it can be challenging to discriminate subpopulations on the basis of function (Sica and Mantovani, 2012). We (Rodero *et al.*, 2012) and others (Movahedi *et al.*, 2010) have used both Ly6c and MHCII expression levels to discriminate macrophage populations, which in the context of wound healing and tumor progression exhibit distinct recruitment patterns and transcriptomic profiles. Specifically, using these markers in UVR-induced inflammation, we could identify two distinct populations among the Ly6c^{low} macrophages, with distinct recruitment patterns. It remains, however, unclear whether Ly6c^{low} MHCII^{low} and Ly6c^{low} MHCII^{hi} arise from distinct myeloid precursors or instead reflect distinct levels of activation in cells that have a common precursor. Although we cannot exclude minor roles for other populations, our results strongly suggest that the melanocyte response is due to the activity of Ly6c^{low}MHCII^{hi} macrophages. This is supported by the correlation between increased Ly6c^{low}MHCII^{hi}

population and melanocyte number over time in several different strains of mice. Moreover, our data further suggest that the CD11c -negative fraction of this Ly6c^{low}MHCII^{hi} population is enough to promote the melanocyte response, as we do not observe any effect after CD11c^{+} cell depletion. This particular macrophage population can also promote tumor growth by inducing T-cell immunosuppression (Movahedi *et al.*, 2010). Altogether, our results imply that neonatal UVR results in the recruitment of a population of macrophages that promote melanocyte proliferation and possibly tumor progression (Zaidi *et al.*, 2011), although the latter needs to be formally demonstrated.

The use of clodronate liposomes has clarified the importance of macrophages in a range of disorders. In our model, clodronate liposomes depleted macrophages without affecting other myeloid populations such as neutrophils or mast cells. Liposome uptake is known to essentially target highly phagocytic cells. As shown in our results, it did not affect all macrophage populations equally, and the depletion was not complete. This could explain the incomplete suppression of the melanocyte response. Alternatively, other parallel mechanisms independent of macrophages might exist to promote the melanocyte response to UVR in neonates. However, the lack of effect on this response observed after depletion of CD11c^{+} cells clearly excludes a role for Langerhans and dermal dendritic cells.

Having demonstrated the importance of macrophages, we next sought to understand the mechanisms involved in the neonatal melanocyte response. We did not observe any defect in the melanocyte response to UVR in *IFNγ*$^{-/-}$ or in *TNFα*$^{-/-}$ mice, suggesting that it is not the classical T helper type 1 inflammatory cytokines that link macrophage and melanocyte responses to UVR.

TLRs are an important pathway in innate immunity and macrophage activation. The increased expression of defensins in the epidermis in our gene expression array also pointed to these receptors as a way of activating recruited macrophages. The increased expression of IL-33, an IL-1 family member, could represent an alternative. All TLRs, as well as the IL-33R, signal through MyD88 and/or TRIF. The normal melanocyte response in *MyD88/TRIF*$^{-/-}$ mice, as well as in ST2 (*IL33R*$^{-/-}$) mice, clearly excludes the importance of these pathways. Finally, we observed a small but significant reduction of the melanocyte response in *IL17*$^{-/-}$ mice. Interestingly, Kotobuki *et al.* (2012) recently proposed a role for IL-17 in the inhibition of human melanocyte activity. They showed that direct treatment of human melanocytes with IL-17 reduced MITF (microphthalmia-associated transcription factor) and Bcl-2 (B-cell lymphoma 2) expression and diminished pigment production activity. However, in our setting, IL-17 may help activate melanocyte activity, at least in terms of proliferation or migration, which Kotobuki *et al.* (2012) did not measure. In addition, our data suggest an indirect role for IL-17 in the context of UVR-induced melanocyte activation. As IL-17 receptors are widely expressed, it is not clear as to how IL-17 mediates its effect; it could be at any level from epidermal cell activation to the recruitment and activation of macrophages.

The lack of effect observed in the knockout mice mentioned above does not imply that these pathways are not involved in the melanocyte response, rather that they are not exclusive and necessary. This may be one reason why our study is not suggestive of an IFN-γ-mediated mechanism driving the melanocyte response, as reported by Zaidi *et al.* (2011). Other differences between their study and ours include the postnatal timing of UVR exposure (P1 vs. P3 in our study), the time between exposure and melanocyte counting (D7 vs. D4 in our study), the mouse strain background (FVB vs. C57BL/6J in our study), and the mode of blocking IFN signaling within the skin (anti-IFN-γ antibody vs. IFN-γ-knockout mice). Despite these differences in the implicated cytokine (possibly due to the caveats just mentioned), both studies point to a critical role of macrophages in the melanocyte response to UVB used at ostensibly equivalent doses, and both have excluded the capacity of UVA to provoke a similar response (Walker *et al.*, 2009; Zaidi *et al.*, 2011).

We do not believe that the activity of macrophages can explain all of the melanocyte proliferation. Other cell types within the skin, particularly keratinocytes, can have a marked influence on melanocyte behavior, including their positioning in the epidermis. This has been shown in mouse models carrying specific mutations. For instance, mutation of *KRT2E* or *EGF* (Fitch *et al.*, 2003), *Integrin β1* (López-Rovira *et al.*, 2005), or *β-Catenin* (Rabbani *et al.*, 2011) in keratinocytes can also increase epidermal melanocyte density. The mechanism involved in the latter case is proposed to involve increased keratinocyte EDN1 expression that may drive melanocyte proliferation or migration. Overexpression of keratinocyte KITL (or stem cell factor) also supports the localization of melanocytes in the murine interfollicular epidermis, either by transgenic overexpression of the membrane-bound form (Kunisada *et al.*, 1998) or indirectly by upregulation of p53, which increases KITL transcription in the epidermis (Box and Terzian, 2008). FGF2 upregulation can also induce the activation of melanocytes (Weiner *et al.*, 2007). However, we did not observe a significant upregulation of endothelins, KITL, or FGF2 in our expression array analysis of the epidermis after UVR. The chemokine CXCL12 (chemokine (C-X-C motif) ligand 12) regulates the migration of melanocyte progenitors downward from the stem cell niche during anagen (Belmadani *et al.*, 2009), but there is no evidence to date that it influences our observed upward migration in the neonatal setting in response to UVR. The above-mentioned molecules that either emanate from keratinocytes or influence keratinocyte differentiation status are among the very few shown to promote melanocyte migration and proliferation *in vivo*. We note that prostaglandin E synthase is upregulated at all time points. Prostaglandin E2 and prostaglandin F2a released from keratinocytes have been shown to stimulate dendrite formation in melanocytes in culture (Scott *et al.*, 2004), suggesting a possible mechanism to investigate in future experiments.

The melanocyte proliferation and migration in neonates in response to UVR (Walker *et al.*, 2009) is not seen in adult skin after a single comparable dose of UVR, and hence is

ostensibly specific to the neonatal periods when UVB exposure is known to markedly exacerbate MM development in mice (Noonan *et al.*, 2001). We suspect that this is because neonatal melanocytes are extremely sensitive to UVR, as chronic UVR seems to be necessary to increase the number of epidermal melanocytes in adult mice (Aoki and Moro, 2005). Innate responses of the skin to neonatal UVR may provide useful information for MM given that the most common subtypes (nodular or superficial spreading MM) appear to be associated with acute sunburns (Gandini *et al.*, 2005), particularly those associated with childhood sun exposure (Whiteman *et al.*, 2001). Although neonatal mouse skin may represent a somewhat specialized system, a single burning dose of UVR to human skin also promotes a strong influx of macrophages (Meunier *et al.*, 1995). Additional evidence links the melanocyte response with carcinogenesis. The number of active melanocytes in 2-week-old mouse epidermis is greatly increased by chemical carcinogen application, and the more carcinogenic the compound, the greater the effect (Iwata *et al.*, 1981). Strong liver carcinogens that are not metabolically activated in skin were ineffective. The compound most effective in inducing melanocyte proliferation was 7,12-dimethylbenz(a)anthracene, a very potent skin carcinogen. Thus, the melanocyte activation is driven either by UVR or by compounds that induce adducts within the DNA of skin cells, both of which can induce melanoma (Iwata *et al.*, 1981; Sharov *et al.*, 2003).

To conclude, in this work, we have functionally verified the important role of macrophages in the melanocyte response to UVR. Using mice genetically deficient for a range of pathways and cytokines, we establish that the melanocyte response is not dependent upon the TNF-α, IFN-γ, IL-33R, or TLR ligand activation pathways, but can be reduced by ablation of IL-17. After a detailed examination of the macrophage populations infiltrating neonatal skin after UVR, we have established that the CCR2-independent recruitment of $Ly6c^{lo}MHCII^{hi}CD11c^{-}$ macrophages is positively correlated with the extent of the melanocyte response. We believe that this work may have important implications in terms of establishing the type of immune environment required for UVR-mediated promotion of melanoma (Wolnicka-Glubisz *et al.* 2007; McGee *et al.*, 2011).

MATERIALS AND METHODS

UVR treatments

Treatments were performed as previously described (Walker *et al.*, 2009). Full details are available in the Supplementary Methods online.

Immunofluorescence and flow cytometry staining

Immunofluorescence staining was performed as reported in Walker *et al.* (2009), and flow cytometry as described in Rodero *et al.* (2012). Full details are available in the Supplementary Methods online.

Skin collection and RNA extraction

After UVR exposure, three pups were killed at each time point. The epidermis was separated from the dermis by floating them in a mixture of thermolysine and $CaCl_2$ in PBS at 37 °C for 2 hours. The epidermis was transferred immediately to RNAlater (AMBION, Austin, TX) and stored at −20 °C before use. The RNA extraction was carried out according to the Qiagen RNeasy Mini protocol (QIAGEN, Mannheim, Germany).

Mouse expression array

From each epidermis, 500 ng of total RNA was used as the starting material to produce cRNA, according to the Illumina TotalPrep RNA Amplification protocol (Illumina, San Diego, CA). From each sample, 1,500 ng of cRNA was hybridized to Illumina MouseWG-6 v2.0 Expression BeadChips (Illumina) and then scanned. Data were extracted using GenomeStudio (Illumina, San Diego, CA) and then imported into GeneSpring GX 11.5.1 (Agilent Technologies, Santa Clara, CA), before normalization using the quantile algorithm and subsequent analysis.

Clodronate treatment to specifically deplete macrophages

Clondronate (Cl2MDP, a gift of Roche Diagnostics GmbH, Mannheim, Germany) or PBS was encapsulated in liposomes containing phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma-Aldrich, St Louis, MO), as described previously (Van Rooijen and Sanders, 1994). Pups were injected with 5 mg ml^{-1} clodronate (clodronic acid)- or PBS (control)-filled liposome suspension, 30 µl intraperitoneally and 30 µl subcutaneously, on the day before UVR (D − 1), the day of UVR (D0), and 2 days after UVR (D2) exposure. Skin was harvested on D3.

Knockout mice

The knockout mice used in this study were as follows: $CCR2^{-/-}$ (Boring *et al.*, 1997), $IFN\gamma^{-/-}$ (Dalton *et al.*, 1993), $IL17^{-/-}$ (Nakae *et al.*, 2002), ST2 ($IL33R^{-/-}$) (Townsend *et al.*, 2000), $MyD88/TRIF^{-/-}$ (Hoebe *et al.*, 2003), $RAG1^{-/-}$ (Mombaerts *et al.*, 1992), and $TNFa^{-/-}$ (Pasparakis *et al.*, 1996). All mice, as well as WT controls, were on a C57BL/6J background. All animals are kept in a modern Optimice caging system (Centennial, CO), under the care of trained animal handlers. The work was approved by the Queensland Institute of Medical Research Animal Ethics Committee (A98004M).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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